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Regulation of environmental factors affecting ethanol & phenolic contents of Jamun wine and in-vitro evaluation of its anti-bacterial efficacy

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***Abstract*— The present study was designed to optimize the environmental factors that determine ethanol levels of Jamun wine. The studied factors included varied levels of total soluble solids, pH, temperature and inoculum size. It was observed that a total soluble solids content of 25° Brix, pH of 4.5, temperature of 25° C and an inoculum size of 10 % (v/v) were best for achieving maximum ethanol levels. With these conditions, the observed bio-chemical structure of wine included an ethanol content of 7.5 % (v/v) with a pH of 3.4, titratable acidity of 0.58 gTartaric acid/100mL, reducing sugars content of 1.2 % (w/v) and total phenolic capacity of 372 µg/mL in terms of gallic acid equivalents. Further, the wine was observed to possess inhibitory activities against common food borne pathogens *E. coli, S. aureus* and *S. typhi*.**

Keywords*— Jamun, wine, TSS, pH, temperature, Inoculum, anti-bacterial efficacy, E. coli, S. aureus, S. typhi.

INTRODUCTION

Jamun, also known as *Syzygium cumini* or Indian blackberry is one of the widely used plants for diabetic treatments by traditional practitioners. It is also used against numerous other medical conditions including microbial infection, oxidative stress, diarrhoea, ulcers and cardiovascular complications (Chagas et al., 2015; Swami et al., 2012). Jamun berries are a famous fruit in South-east Asia and are consumed as such or in the form of jams, jellies, vinegar, syrup and wine (Swami et al., 2012). There are a few reports on fermentation of Jamun berry pulp into wine (Joshi et al., 2012; Chowdhary and Ray, 2007; Patil et al., 2012). However, the effect of environmental factors on physico-chemical structure of Jamun wine has not been studied.

Further, owed to the increase in lifestyle based disorders occurring due to poor physical exercise and improper nutrition, the concept of functional foods is on rise (Corbo et al., 2014; Chauhan et al., 2015). The pharmaceutical properties of these foods are ascribed to their phytochemical and bio-active constituency, which in turn are affected by processing and manufacturing procedures. Therefore, it becomes necessary to develop and produce functional foods in best suited conditions, so that their nutritional status can be enhanced.

In view of the above mentioned factors, growing consumer interest in phytotherapy and owing to the medicinal properties of Jamun, the present study was undertaken to optimize environmental conditions for fermentation of Jamun pulp and *in-vitro* evaluation of the efficacy of Jamun wine against common food borne pathogens *Salmonella typhimurium, E.coli* and *Staphylococcus aureus* as discussed at length.

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MATERIALS AND METHODS

A. Microorganisms

Yeast strain used in the present study, *Saccharomyces cerevisiae* MTCC 786 was acquired from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India.

B. Jamun juice extraction

Jamun berries were steamed for 20 min and deseeded mechanically by hands. The pulp was crushed in a blender and water was added to the must obtained in a ratio of 1:1. The extract so prepared was stored at 4°C prior to fermentation.

C. Inoculum preparation

For the preparation of inoculum, 25 ml of sterilized MYPD broth (yeast extract, 0.3%, malt extract, 0.3%, peptone, 0.5% and dextrose, 1% (w/v), pH 4.5) was inoculated with *Saccharomyces cerevisiae* MTCC 786 and incubated at 22±2˚C on a rotary shaker (150 rpm) overnight. Pre-inoculum was prepared by separating the cells by centrifugation at 10000 rpm (4^{o}C, 15 min) and washed twice, followed by re-suspension in normal saline to raise a final concentration of 10^8 cells/ml. The inoculum was prepared by transferring 10 ml of the pre-inoculum to 100 ml of Jamun extract whose TSS was adjusted to 5°B with the help of refractometer (Erma, Japan) and incubated overnight on a rotary shaker at 22±2˚C. This was then used for further experiments.

D. Production media and fermentation conditions

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For each treatment, 100 mL Jamun extract was taken in different sets of 250 mL Erlenmeyer flasks. Cane sugar was added to adjust the TSS at 20°B and pH was adjusted to 5 with citric acid and 100 ppm sodium metabisulfite was added to it. These flasks were inoculated with a 10% (v/v) inoculum made with an overnight grown culture of *S. cerevisiae* having a viable cell count of 1×10^8/mL and were cotton plugged afterwards. Fermentation was carried for 10 days at 28±2°C, unless otherwise stated. After completion of fermentation, the wine was siphoned and stored at 4°C for further analysis.

D. Standardization of environmental factors

This was done by studying the effect of incubation temperature, pH of the medium, inoculum size and sugar concentration in the medium by one factor at a time (OFAT) approach. Under each set of experiments, only the factors studied was varied and rest of the conditions were kept constant as mentioned in the previous section.

1. The effect of TSS was studied by adjusting the sugar content in the production media at 10, 15, 20 and 25 and 30° B with cane sugar.
2. The effect of pH was studied by varying the pH (3.2-6.0) of the production media using citric acid or sodium bicarbonate.
3. The effect of temperature was studied by incubating the extracts based production media, after inoculation, at different temperatures including 15°C, 20°C, 25°C, 30°C, 35°C and 40°C.
4. The effect of inoculum level was studied by inoculating the production media with varying levels of inoculums (2-12% v/v) having 1×10^8 viable cells/mL.

E. Analysis of the wine

Physio-chemical analysis of the wine was carried out by using standard protocols. The parameters studied included ethanol content (Caputi et al. 1968), titratable acidity (AOAC), total phenolic content (Rathee et al. 2006).

F. Production of Jamun wine under optimized conditions

Following the initial standardization of the environmental factors, the best suited conditions were employed for the production of Jamun wine. For this, a TSS of 25° Brix, pH 4.5 and inoculum size of 10 % (v/v) was added to the 100 mL of Jamun extract taken in 250 mL Erlenmeyer flask. This was followed by supplementation of the media with 100 ppm sodium metabisulfite. The flask was then cotton plugged and incubated at 25°C in a BOD incubator at stationary phase for 20 days. After fermentation, the wine was siphoned and filtered and was racked for repeatedly for 3 times in a time interval of 7 days. It was then stored in an amber colored glass bottle at 4°C for further use.

G. Organoleptic evaluation of Jamun wine

Sensory evaluation of Jamun wine was done by a panel of 10 judges. A 30 point scale method was used for analysis of appearance, aroma, sour, taste, after taste and overall impression.

H. Anti-bacterial efficacy of Jamun wine

Antibacterial activity of prepared wine was evaluated against *S.* Typhimurium, *S. aureus* and *E. coli* by well diffusion technique (Deans and Ritchie, 1987). The actively grown cultures with cell count of 1×10^5 cfu/mL were selected for plate assay. 100 µL the respective liquid culture was spread on nutrient agar plate to create a bacterial lawn. Two wells with diameter of 6 mm were punched in each nutrient agar plate and 100 µL of Jamun wine added to one of the wells in each plate under aseptic condition. As a positive control, 100 µL of 10% (v/v) pure ethanol were also loaded separately in the other well in each plate. The plates were left for 30 min at room temperature for the diffusion of the test samples before incubating at 37°C for 24 h after which the diameter of zones of inhibition were measured. The analyses were carried out in triplicates.

RESULTS AND DISCUSSION

Fermentation of Jamun pulp yielded a clear purple coloured wine with an ethanol content of 5 % (v/v), pH 3.8, titratable acidity of 0.57 gTartartic acid/100mL, total reducing sugars of 3.4 g/100 mL and total pheolic content of 372 µg/mL. Earlier also Jamun wine with similar composition has been reported [3-5].

A. Effect of TSS on Jamun wine fermentation

With an increase in TSS, ethanol levels and total phenolic content (TPC) were observed to gradually increase up to 25° Brix, after which ethanol levels started decreasing with any further increasing in TSS levels of the media (Fig. 1a). Maximum ethanol level of 7.2% (v/v) and a TPC of 478.3 µg/mL were observed when initial TSS of media was 25° Brix, followed by 6.5 % (v/v) ethanol and 460.3 µg/mL TPC, with media having an initial TSS of 20° Brix. Minimum ethanol levels of 3.5 % (v/v) were observed in case of TSS 10° Brix and a TPC of 408.2 µg/mL was observed in this case. This may be due to that fact that lower sugar concentration was not enough for maximum ethanol production. Decrease in ethanol with increase in TSS beyond 25° Brix can be ascribed to the development of hypertonic environment which was not favourable for the growth of the yeast. Further, direct correlation of phenolic with ethanol shows that ethanol

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majorly contributes to their extraction into the media. Higher ethanolic concentrations led to higher phenolic extraction. These observations are in correlation with earlier studies which have shown a TSS of 24±1° Brix to be optimum. Maximum levels of ethanol have been reported at 24° Brix in case of litchi, strawberry, kinnow and carambola wine also [12-15].

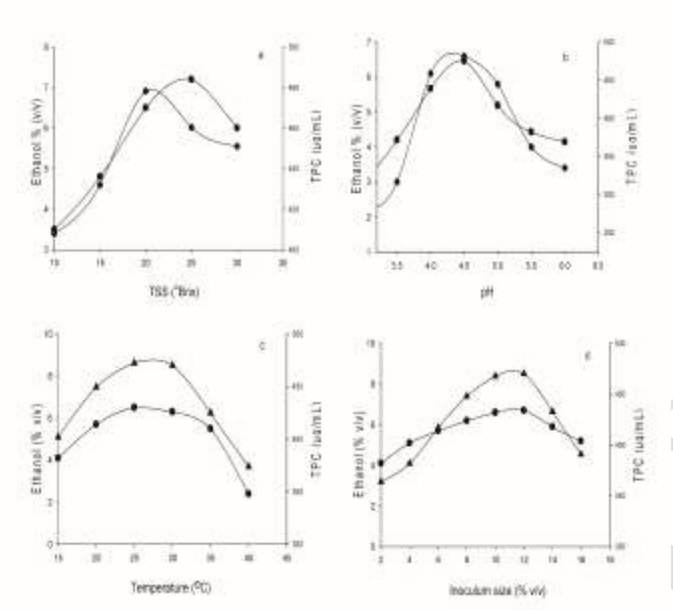

Fig. 1 Effect of TSS (a), pH (b), temperature (c) and inoculum size (d) on ethanol level (●) and total phenolic content (▲) of Jamun wine.

B. Effect of pH on Jamun wine fermentation

The best pH for the production of Jamun pulp wine was observed to be 4.5 with the ethanol level being 6.6% (v/v) with a TPC of 475.2 µg/mL followed by 6.1% (v/v) ethanol and 439 µg/mL TPC at pH 4.0 (Fig. 1b). Lowest ethanol level and TPC of 2.2% (v/v) and 315.4 µg/mL, respectively were observed at pH 3.0. At highest pH of 6.0, the ethanol level was found to be 3.4% (v/v) and TPC was 369 µg/mL. These observations are in agreement with the earlier reports by Paul and group (2014), who also found the pH level of 4.5 to be the optimum for the production of sweet potato wine and obtained an ethanol level of 9.6% (v/v). In case of jackfruit wine, a pH of 4 was reported to be suitable as compared to the pH 5 and 6 [16]. Sevda and Rodrigues, observed that with increase in pH from 2.5 to 5.0 there was gradual increase in rate of fermentation and recorded pH 4 to be optimal for guava must fermentation [17].

Reduced ethanol levels in low pH and high pH causes chemical stress on yeast and therefore, they are not able to grow and thrive at an optimum rate. Abnormal pH causes denaturation of enzymes also, which ultimately hinders the metabolic processes of the microbe [18].

C. Effect of temperature on Jamun wine fermentation

The best temperature for the production of Jamun pulp wine was observed to be 25°C. An ethanol level of 6.5% (v/v) and a TPC of 472.4 µg/mL were attained at 25°C followed by 6.3% (v/v) ethanol and 449.4 µg/mL at 20°C (Fig. 1c). Lowest ethanol level of 2.4% (v/v) was observed at 40°C with a TPC of mere 374 µg/mL .At 15°C, the ethanol level was found to be 4.1% (v/v) with a TPC of 402.4 µg/mL. Our observations are in agreement with earlier reports which have also show a temperature of 25°C to be optimum for the production of litchi, plum, tomato and apple mango wine production [12, 19-21]. It is interesting to note that as compared to lower temperature ranges, the ethanol yields were lesser at higher temperatures. This is due to decreased ethanol tolerance of yeast at higher temperatures. It is well known that fermentation over 35°C leads to premature end of fermentation and therefore, lower ethanol rates are achieved [22]. During fermentation, the presence of ethanol may add-on to the destructive effects of high temperatures. Under these conditions, the growth of yeast cells is limited and their nitrogen assimilation becomes poorer. The tolerance to ethanol decreases, ultimately, the fermentation process gets stuck. As a result, the correct proceeding of the fermentation is partly related to the maintenance of a non-elevated temperature during the process [23]. Lower temperatures however, increases the ethanol tolerance of yeast due to unsaturated fatty acid residues in the plasma membrane. This is why the viable cell count at the end of the fermentation is more at lower temperatures as compared to the higher ones [24].

D. Effect of inoculum size on Jamun wine fermentation

For a good fermentation, inoculum size is an important factor. Too low or too high number of yeast cells may lead to stunted results. A direct correlation between inoculum size and ethanol level and TPC was observed in the production of Jamun pulp wine. Best level of ethanol was observed at 10% seed volume. 6.6% (v/v) ethanol and 467.8 µg/mL TPC were found to be the maximum followed by 6.2% (v/v) and 420.04 µg/mL TPC in the media inoculated with 8% seed culture (Fig. 1d). Any decrease in inoculum size led to decreased production of ethanol in the media. Singh and Puyo [25] observed a similar correlation between inoculum size and ethanol levels. 10 % (v/v) inoculum size was reported as optima for the production of guava wine and it yielded an

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ethanol level of 5.6 % (v/v). They also reported 10 % inoculum as optimum for the production of other wines including jamun, apple, pear and plum. An inoculum level of 10% (v/v) had been reported optimum for the alcoholic fermentation of pear juice and guava juice [26,27]. Similar results have been reported by Singh *et al* and Singh and Kaur for the production of lichi and kinnow wine [14, 12].

E. Bio-chemical analysis of Jamun wine produced under optimized conditions

An ethanol content of 8.5% (v/v) was observed with a pH of 3.4, titratable acidity of 0.58 gTartaric acid/100mL and reducing sugars were found to be 1.2 % (w/v). Total phenolic capacity of the wine was found to be 489 µg/mL in term of gallic acid equivalents. Joshi and co-workers [3] also reported the production of Jamun wine in similar set of environmental conditions and the overall structure of the wine similar to that of our wine. Another study has described the production of wine from Jamun and an ethanol content of 8.82% (v/v) after inoculation of media with *Saccharomyces cerevisae* {10% (v/v)} with a pH of 3.25 and acidity of 0.61 gTartaric acid/100 mL was observed [5]. Chowdhary and Ray [4] also produced wine from fruit pulp and it was found to possess an ethanol content of 6% (v/v) and a titratable acidity of 1.1, with a pH of 4.5.

F. Maturation of Jamun wine

As expected, the bio-chemical constituency of the wine changed during maturation. Ethanol content after one year of maturation was found to be 8.1 % (v/v). A pH of 3.1, titratable acidity of 0.55 gTartaric acid/100mL and reducing sugars were found to be 1.5 % (w/v). TPC content of the wine was found to be 474 µg/mL (Table 1). This observation is in complete agreement by the report of Joshi and co-workers who also confirmed the bio-chemical changes in Jamun wine after one year of maturation. The reduced levels of ethanol may be due to reaction between ethanol and acids to form esters. Changes in titratable acidity can be attributed to the precipitation of acids into salts. Increase in reducing sugars may be due to hydrolysis of non-reducing sugars into reducing sugars. The decrease in polyphenolic content may be due polymerization, condensation, degradation and precipitation of phenolic compounds, with time [3].

TABLE 1. EFFECT OF MATURATION ON BIO-CHEMICAL STRUCTURE OF JAMUN WINE

Parameter	**Before maturation**	**After 12 months of maturation**
Color	Light purple	Dark purple
Ethanol % (v/v)	8.5	8.1
pH	3.4	3.1
Reducing sugars (g/100mL)	1.2	1.5
Titratable acidity (gTartaric acid/100mL)	0.58	0.55
Total phenolic content (µg/mL)	489	474

G. Sensory evaluation of Jamun wine

An acceptable wine is perceived to be composed to good wine in terms its taste, aroma, after-taste, body and overall acceptability. Matured wine scored better than that of un-matured wine. Table 2 depicts the results of sensory evaluation of Jamun wines. Both the wines were found to be acceptable in sensory evaluation. However, unmatured wine was found to be inferior in terms of mouth-feel and taste. These observations are in agreement with Joshi and co-workers [3], who showed good sensorial properties of Jamun wine after maturation. Chowdhary and Ray [4] have also shown good sensory grades of Jamun wine. However, commercial wines scored better than that of Jamun wine . Similar observations were also reported by Patil and group [5], who showed Jamun wine's acceptability in sensory evaluation.

TABLE 2. SENSORY EVALUATION OF JAMUN WINE

Parameter	**Unmatured wine**	**Matured wine**
Appearance	3	5
Aroma	3	5
Sour	3	4
Taste	3	5
After-taste	3	5
Overall impression	3	5
Total	19	29

Anti-bacterial efficacy of Jamun wine

Jamun wine showed *in-vitro* antibacterial activity against the common pathogens tested in the study. The zones of

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inhibition for *S. typhimurium*, *S. aureus* and *E. coli* and were found to be 10.2±0.12 mm, 09±0.18 mm and 07±0.14 mm, respectively (Table 3). On a contrary, zone of inhibition produced by 10 % (v/v) ethanol were found to be smaller as compared to wine. This can be attributed to the presence of organic acids, low pH and phytochemical compounds in the wine. Earlier also it has been shown that different combinations of pH and ethanol have produced similar results against *Helicobacter pylori* [28]. The presence of these bio-chemical components in wine may have acted synergistically in inhibiting the pathogenic microbes. Anti-bacterial efficacy of Jamun is not a new concept. Previously also, Jamun fruit extract and its peel have shown considerable anti-microbial activities [29-31]. Oliviera and co-workers [32] have shown anti-microbial potency of Jamun leaves extract. Seed and stem extracts have also exhibited inhibiting activities against pathogenic bacteria [33,34].

TABLE.3 ZONES OF INHIBITION (MM) OF JAMUN WINE AND 10% ETHANOL (CONTROL) AGAINST *SALMONELLA TYPHIMURIUM*, *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI*.

Pathogen	Jamun wine	10 % (v/v) Ethanol
S. typhimurium	8	0
E. coli	12	0
S. aureus	10	0

CONCLUSION

The observations from the present study indicate that environmental factors play a crucial role for ethanol levels of Jamun wine. A TSS of 25° Brix, pH 4.5, inoculum size 10 % (v/v) and temperature of 25° C are the optimized intensities of these factors for Jamun wine. Rich phenolic content and anti-bacterial of this beverage against the common food borne pathogens *E. coli, S. aureus* and *S. typhi* indicates its pharmacological importance. *In-vivo* studies regarding further evaluation of medicinal potencies of Jamun wine is therefore suggested.

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